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Toward Peptide Nucleic Acid (PNA) Directed Peptide Translation Using Ester Based Aminoacyl Transfer

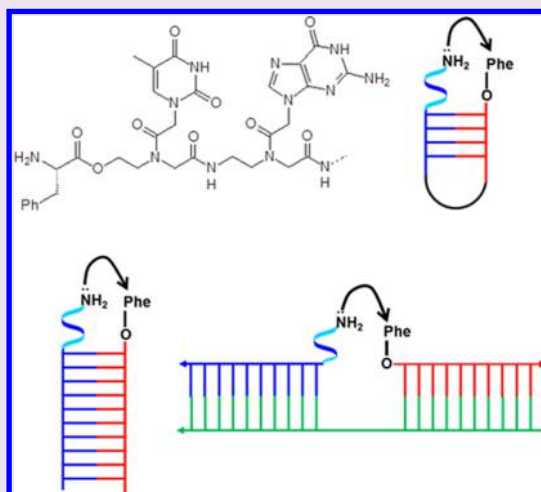
Abhishek Singhal,^{†,||} Valentina Bagnacani,^{§,⊥} Roberto Corradini,[§] and Peter E. Nielsen^{*,†,‡}

[†]Department of Cellular and Molecular Medicine and [‡]Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, The Panum Institute, Blegdamsvej 3c, DK-2200 Copenhagen, Denmark

[§]Department of Chemistry, University of Parma, Parco Area Delle Scienze 17/a, 43124 Parma, Italy

S Supporting Information

ABSTRACT: Peptide synthesis is a fundamental feature of life. However, it still remains unclear how the contemporary translation apparatus evolved from primitive prebiotic systems and at which stage of the evolution peptide synthesis emerged. Using simple molecular architectures, in which aminoacyl transfer of phenylalanine occurs either between two ends of a PNA stem loop structure, between two PNAs in a duplex, or between two PNAs assembled on a PNA template, we show that *bona fide* template instructed phenylalanine transfer can take place. Thus, we have identified conditions which allow template assisted intermolecular aminoacyl transfer using simple ester aminolysis chemistry primitively analogous to the ribosomal peptidyl transferase reaction in the absence of anchimeric assistance from ribose and ribosome catalysis. These results help define the minimum chemical boundary conditions for the translation process and also give insight into the possibilities for the prebiotic emergence of RNA-independent translation.



Nucleic acid instructed peptide synthesis is one of the fundamental features of the central dogma of molecular biology of life. However, it still remains unclear how the contemporary translation apparatus evolved from primitive prebiotic systems and at which stage of evolution peptide synthesis emerged. The RNA world hypothesis implicates that translation is a later evolutionary event as supported by the demonstration of ribozyme catalyzed amino acid transfer reactions^{1,2} and peptide bond formation by *in vitro* selected ribozymes.^{3,4} Furthermore, it has been demonstrated that peptidyl transfer reactions can be mediated by an aminoacyl tRNA mimic, in which imidazole-catalyzed peptide synthesis was dependent on sequence complementarity between a 3'-CCA sequence of an RNA minihelix and a puromycin containing oligonucleotide precursor, without the involvement of ribosomes or rRNA.^{5,6} Most importantly, the amino acid had to be delivered by a ribose and not a deoxyribose at the 3'-end of the RNA donor, thereby stressing the necessity for anchimeric assistance of a vicinal hydroxyl group for acyl transfer. Finally, from a synthetic chemistry approach more sophisticated DNA-templated multi-step synthesis of oligo peptides (amides) have been devised exploiting *in situ* chemical activation or a variety of active ester derivatives.^{7–17} Most recently, a DNA coded translation system using complex PNA pentamer macrocyclic adaptors as “tRNA mimetics” was developed based on click chemistry coupling.¹⁸

In general, peptides and peptide-like molecules appear the more compatible with prebiotic synthesis and conditions than

RNA. In this context, the pseudopeptide nucleic acid mimic PNA (peptide nucleic acid) has been proposed as a model for a robust prebiotic evolutionary predecessor of RNA being capable of chemical sequence information transfer from one PNA oligomer to another (a replicative process)¹⁹ as well as from a PNA oligomer to an RNA oligomer (a PNA to RNA transition).^{20,21} Furthermore, aegPNA building blocks have been identified in “prebiotic soup” experiments,²² and precursors for aminobutyric acid or ornithine based PNA oligomer²³ have been identified in meteorites.²⁴ Most interestingly, examples of pyrimidyl amino acid derivatives have been reported as plant secondary metabolites,^{25–27} and N-(2-aminoethyl)glycine, the backbone of PNA, was found in cyanobacteria.²⁸ Thus, some evidence for PNA-like chemical structures also exists in contemporary biology. In order to evaluate further the possibility of involvement of non-RNA oligomers such as PNA in prebiotic emergence of life,²⁹ and also to study potential prebiotic translational processes in such evolution, we have designed and characterized simple systems capable of performing PNA sequence-directed amino acyl transfer reactions, primitively mimicking the tRNA/mRNA/ribosomal translation but without ribose and ribosome catalysis.

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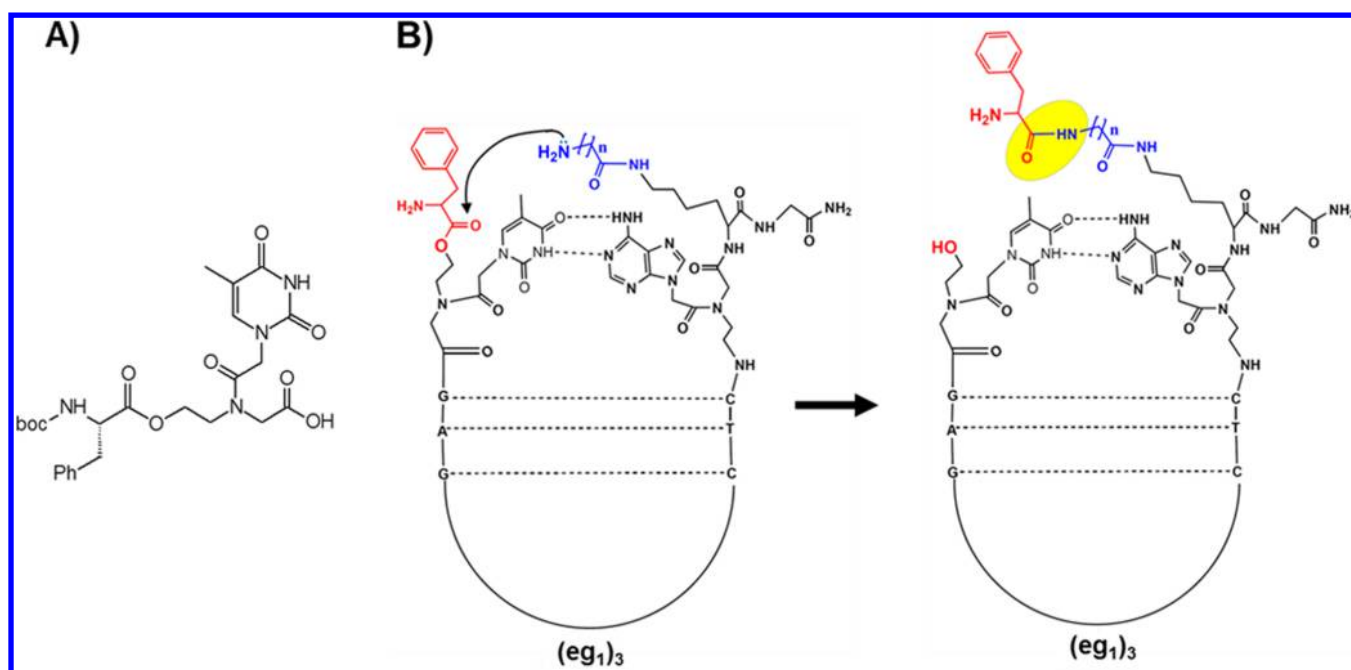


Figure 1. (A) Structure of phenylalanine ester PNA monomer, (B) Schematic structure of the PNA hairpin construct for phenylalanine interstrand transfer. In the model construct, amino-acylated PNA provides the NH_2 group that can attack the ester linkage of the phenylalanine moiety at the other end of the hairpin to form a peptide bond (yellow circle) (the loop of the hairpin is formed by an ethylene glycol type linker (eg_1 : 8-amino-3,6-dioxaoctanoyl)).

In the present work, we focus on studying the acyl transfer process using a minimal chemical model system based on aminoacylated PNA oligomers using simple ester chemistry without anchimeric assistance, in which PNA serves as a model for nonribose nucleic acids. PNA oligomers with a 2-hydroxyethylamino terminus chemically “charged” with an amino acid through an ester linkage were prepared, and the transfer of this amino acid to an amine acceptor on another PNA oligomer assembled by hybridization in a binary duplex or a ternary PNA templated system was studied. We show that under optimized conditions with reduced water activity hybridization dependent interPNA acyl transfer does indeed take place. These results are relevant for the discussion and evaluation of which nucleic acid analogues and mimics may support nucleic acid instructed peptide translation systems based on simple acyl ester chemistry, and thus for evaluating the merits of possible prebiotic chemical scenarios in the origin of life.

RESULTS

A model system was designed in which the donor is a phenylalanine (Phe) ester on an N-(2-hydroxyethyl)glycine thymine unit at the PNA “N-terminal”, and the acceptor is the primary amine of a glycine amide or an alkyl amine at the PNA C-terminal of a PNA four base pair hairpin structure (Figure 1B). To this end, a thymine PNA monomer containing a phenylalanine ester unit replacing the terminal amino group was synthesized (Figure 1A) (Supporting Information). The distance between the primary amine acceptor and the Phe donor was varied by using different linker moieties on the acceptor end (Table 1 and Supporting Information Table S1). In order to identify reaction conditions favoring peptide-bond formation, the hairpin PNA constructs were examined in aqueous buffer at slightly alkaline conditions (pH 9) at 30 °C. HPLC analysis revealed that ester hydrolysis was the major

reaction and no Phe-transfer product could be detected. Thus, in order to search for favorable acyl-transfer reaction conditions, the stability of the Phe-ester PNA 3114 was determined in various buffer systems (CHES, and carbonate (Supporting Information Figure S1)) at different pH (7.5–9) and temperatures (25–45 °C). Not surprisingly, the stability decreased with increasing temperature, whereas pH 7.5–9 and the type of buffer had less influence (Supporting Information Figure S2). In an effort to reduce the rate of ester hydrolysis, an organic cosolvent (DMF, DMSO, NMP, NMAA, HMPA, or dioxane) was added. Because HPLC analysis showed appearances of many (unidentified) side products in the presence of DMF, NMP, HMPA, or dioxane but not when using DMSO or NMAA, we focused on the latter two for further experiments. As anticipated, the Phe-ester PNA showed a highly reduced rate of hydrolysis in the presence of NMAA (as well as DMSO) (Supporting Information Table S2 and Figure S3).

Furthermore, in a parallel experiment PNA acetylation using high excess of ethyl acetate was found to give higher yields in carbonate buffer compared to CHES or imidazole buffer in 60% DMSO (Supporting Information Figure S4 and S5), and using either ethyl acetate or phenylalanine methylester as the acyl donor, we observed a 3-fold higher transfer yield (~9%) for the amino alkyl acceptor PNA (PNA 3844) compared to the glycineamide PNA (PNA 3385) (Supporting Information Figure S6 and S7), which can be ascribed to the lower nucleophilicity of the glycineamide amino group compared to the simple alkylamine.

Phenylalanine Interstrand Transfer in a Hairpin PNA Duplex System. Having established conditions under which aminoacylation of an acceptor amino-PNA by an acyl ester donor can take place, we focused on an intramolecular transfer PNA system composed of a four base pair PNA hairpin in which the transfer would occur from a Phe-ester at the one

Table 1. PNA Constructs

PNA No.	PNA sequence
3747	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys-Gly-NH ₂
3748	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys(β -Ala)-Gly-NH ₂
3749	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys(aminobutanoyl)-Gly-NH ₂
3750	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys(aminohexanoyl)-Gly-NH ₂
3751	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys(aminooctanoyl)-Gly-NH ₂
3871	H-Phe-O-T-AGG-(eg ₁) ₃ -CTCA-Lys(aminooctanoyl)-Gly-NH ₂
3820	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys(Gly-aminooctanoyl)-Gly-NH ₂
3382	H-Gly-(β -Ala)-ACA TCT AGT G-NH ₂
3383	H-Gly-(4-aminobutanoyl)-ACA TCT AGT G-NH ₂
3384	H-Gly-(5-aminopentanoyl)-ACA TCT AGT G-NH ₂
3385	H-Gly-(6-aminohexanoyl)-ACA TCT AGT G-NH ₂
3386	H-Gly-(7-aminoheptanoyl)-ACA TCT AGT G-NH ₂
3387	H-Gly-(8-aminooctanoyl)-ACA TCT AGT G-NH ₂
3844	H-(6-aminohexanoyl)-ACA TCT AGT G-NH ₂
3952	Ac-GTG ATC TAC A-Lys(eg ₁)-Gly-NH ₂
3953	Ac-GTA GTC TCA A-Lys(eg ₁)-Gly-NH ₂
3114	H-Phe-O-T-GTA GAT CAC-NH ₂
3852	H-CGT ATA GAG T-NH ₂
3853	H-(β -Ala)-CGT ATA GAG T-NH ₂
3854	H-(4-aminobutanoyl)-CGT ATA GAG T-NH ₂
3855	H-(5-aminopentanoyl)-CGT ATA GAG T-NH ₂
3856	H-(6-aminohexanoyl)-CGT ATA GAG T-NH ₂
3857	H-(7-aminoheptanoyl)-CGT ATA GAG T-NH ₂
3858	H-(8-aminooctanoyl)-CGT ATA GAG T-NH ₂
3671	H-eg ₁ -GTG ATC TAC AGC ATA TCT CA-Lys-NH ₂
Authentic PNA Products	
3818	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys (Phe-aminooctanoyl)-Gly-NH ₂
3822	H-Phe-O-T-GAG-(eg ₁) ₃ -CTCA-Lys(Phe-Gly-aminooctanoyl)-Gly-NH ₂
3954	Ac-GTG ATC TAC A-Lys(eg ₁ -Phe-H)-Gly-NH ₂
3955	Ac-GTA GTC TCA A-Lys(eg ₁ -Phe-H)-Gly-NH ₂
3977	H-Phe-CGT ATA GAG T-NH ₂
3978	H-Phe-(β -Ala)-CGT ATA GAG T-NH ₂
3979	H-Phe-(4-aminobutanoyl)-CGT ATA GAG T-NH ₂
3980	H-Phe-(5-aminopentanoyl)-CGT ATA GAG T-NH ₂
3981	H-Phe-(6-aminohexanoyl)-CGT ATA GAG T-NH ₂
3982	H-Phe-(7-aminoheptanoyl)-CGT ATA GAG T-NH ₂
3983	H-Phe-(8-aminooctanoyl)-CGT ATA GAG T-NH ₂

end of the PNA to an amino group at the other end of the PNA (Figure 1B, Table 1). Choosing the more reactive alkylamine system (over glycinamide), we analyzed a series of linker moieties (C0, C3, C4, C6, and C8 chain: PNAs 3747–3751), thereby varying the distance between the two reacting groups at the end of the PNA hairpin stem. Computer molecular modeling indicated that the C8 linker (PNA 3751) should be optimal for the ca. 20 Å distance across the end of the hairpin.

HPLC/MALDI-TOF analysis of the reaction of PNA 3751 (90% DMSO in carbonate buffer, pH 9, 50 °C) allowed us to identify and quantify the phenylalanine transfer product (Figure 2A and Supporting Information Table S4). The highest yield (22%) was obtained with the C8-linker PNA (PNA 3751) as compared to PNAs with the shorter linkers (Supporting Information Table S6), and as seen for the reaction with ethyl acetate, the glycine amide acceptor (PNA 3820), (Supporting Information Table S5 and Figure S8) forming a *bona fide* peptide bond is close to 10-fold less efficient than the aminoalkyl acceptor (PNA 3751), despite the higher degree

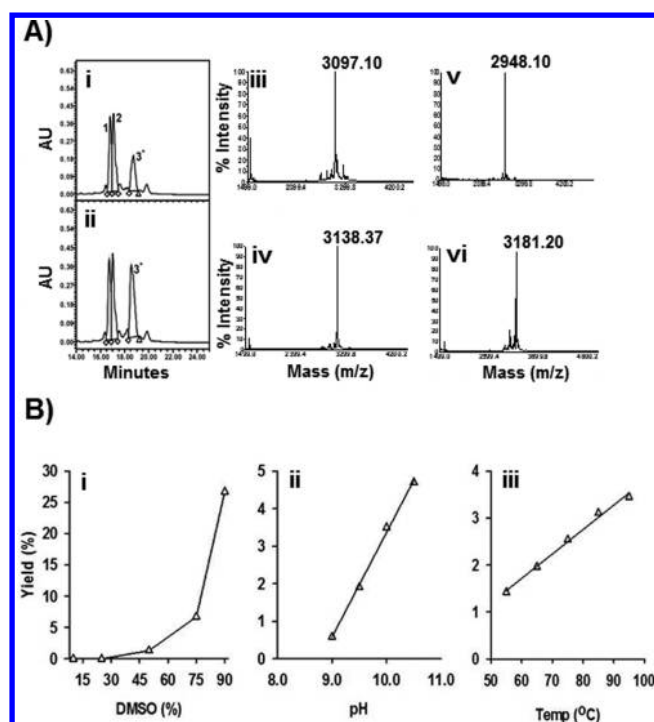


Figure 2. (A) HPLC and MALDI mass spectrometry analysis of reactions in the hairpin PNA 3751 system. Reactions were carried out with 1 mM concentration of hairpin amino alkyl amide PNA 3751 (with C8 linker) in a mixture of DMSO (90%) with 50 mM carbonate buffer, pH 9, at 50 °C for 24 h. Peak 1 corresponds to intact helix PNA, peak 2 corresponds to hydrolyzed ester PNA, and peak 3 corresponds to the Phe-transfer product. (i) HPLC analysis of the reaction mixture. (ii) As part i but with coinjection of chemically synthesized authentic product. (iii) MALDI mass spectrometric analysis of purified product (peak 3). (iv) As part iii but following acetylation. (v) Hydrolysed ester PNA product (peak 2). (vi) Intact hairpin PNA 3751 construct after acetylation. (B) Effect of DMSO, pH, and temperature on the phenylalanine intramolecular transfer in hairpin PNA 3751. Reactions were carried out with 1 mM hairpin amino alkylamide PNA 3751 in a mixture of DMSO in 50 mM carbonate buffer at different pH and temperature. (i) Carbonate buffer at pH 9 at 60 °C for 48 h. (ii) DMSO concentration (90%) and temperature 60 °C for 1 h, and (iii) DMSO concentration (90%) and pH 9 for 1 h. The yield of Phe-transfer is expressed relative to total PNA as measured by HPLC analysis.

of protonation of the alkylamine at pH 9 ($pK_a \sim 8$ for glycine amide and $pK_a \sim 10$ for the alkylamine).

In order to optimize the conditions for intramolecular Phe-transfer, a series of experiments were performed exploring the effect of organic solvent (DMSO 10–90%), pH (8–11) and temperature (50–90 °C). The reaction was strongly and nonlinearly dependent on the DMSO concentration, and no product could be detected below 25% DMSO (Figure 2B,i). Likewise, increased pH (Figure 2B,ii) and temperature (Figure 2B,iii) strongly favored Phe-transfer.

However, increasing temperature as well as pH, and the presence of organic solvents including DMSO, weaken the stability of PNA duplexes,³⁰ and therefore, conditions that favor chemical acyl transfer disfavor hybridization including hairpin formation. Therefore, we measured the thermal stability of PNA 3751 in the presence of DMSO in order to elucidate the importance of the “hairpin structure” for Phe-transfer. The results (Supporting Information Figure S9) show that addition of DMSO very significantly reduces duplex stability, and in 50%

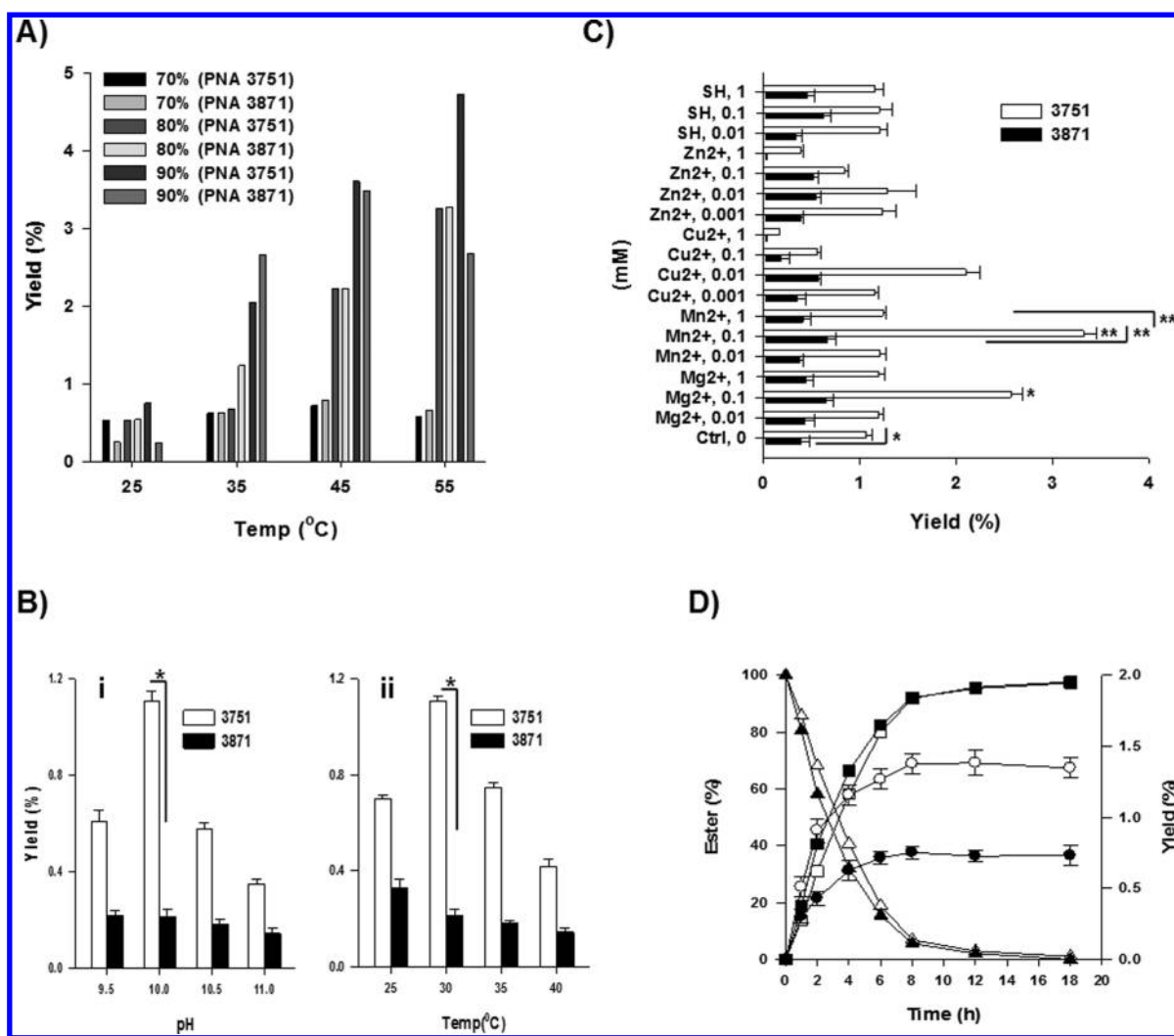


Figure 3. (A) Effect of DMSO concentration and temperature on the Phe-transfer intramolecular transfer in the PNA 3751 hairpin. Reactions were carried out in 50 mM carbonate buffer, pH 9 for 10 h with 1 mM PNA 3751 or with a mis-match control (PNA 3871). (B) Effect of pH and temperature in the presence of NMAA on the Phe-transfer intramolecular transfer in the PNA 3751 hairpin. Reactions were carried out with 1 mM PNA 3751 or with a one mis-match control (PNA 3871) in 50 mM carbonate buffer containing 20% NMAA for 24 h at different pH (and 30 °C) (i) and temperature (pH 10) (ii) (* $p < 0.05$). (C) Effect of metal ions or Ser–His peptide catalyst on the Phe-transfer. Reactions were carried out using different concentrations (0.01–1 mM) of metal cations and dipeptide (Ser–His) under the above experimental conditions for 24 h (* $p < 0.05$, ** $p < 0.001$). (D) Time kinetics analysis of ester hydrolysis and Phe-transfer. The left axis shows the fraction of intact PNA 3751 (open triangle) and PNA 3871 (filled triangle) ester as well as hydrolyzed PNA 3751 (open square) and PNA 3871 (filled square), while the right axis shows the product yield of PNA 3751 (open circle) and PNA 3871 (filled circle) obtained from HPLC analysis. The experiments were performed under the optimized experimental conditions (20% NMAA with 50 mM carbonate buffer, pH 10 at 30 °C). Experiments were performed in triplicate, and yields were expressed as percent (%) yields (bar represent standard error of the mean). p -values were calculated according to Student's t -test.

DMSO, no thermal transition was observed, indicating very low stability of the hairpin duplex under these conditions (Supporting Information Figure S9). These data combined with the observation that the highest yields were obtained at pH, temperatures, and DMSO concentrations, which according to the T_m data are not compatible with stable PNA hairpin duplexes, would indicate that the transfer reaction is not facilitated by the PNA duplex but is rather a consequence of the “intermolecular character” of the reaction. In support of this conclusion, sequence mis-matched hairpin PNA 3871 behaved similarly to PNA 3751 in terms of Phe-transfer yields (Figure 3A), arguing that under these reaction conditions product formation was not dependent on hairpin structure formation.

By exploiting the knowledge gained from the above results, we decided to further examine whether conditions (in terms of reduced temperature/or DMSO concentration/or other

organic cosolvents) could be identified under which the transfer is indeed facilitated by PNA duplex formation. Most interestingly, exchanging DMSO with NMAA was less detrimental to PNA duplex stability (Supporting Information Figure S9). Thus, hairpin duplexes were significantly more stable in NMAA containing carbonate buffer at pH 9.0 as compared to DMSO, and this difference is more pronounced at higher organic solvent content, yielding twice the T_m value at 60% organic solvent.

Consequently, we repeated the experiments using full match (PNA 3751) and mis-match (PNA 3871) PNA hairpins in a mixture of NMAA (20%) in carbonate buffer at different pH and temperature. These data show a clear optimum for the match PNA at pH 10 (Figure 3B,i) at 30 °C (Figure 3B,ii), and most importantly, an up to 6 fold higher yield than for the mismatch PNA (PNA 3871). Thus, we conclude that under

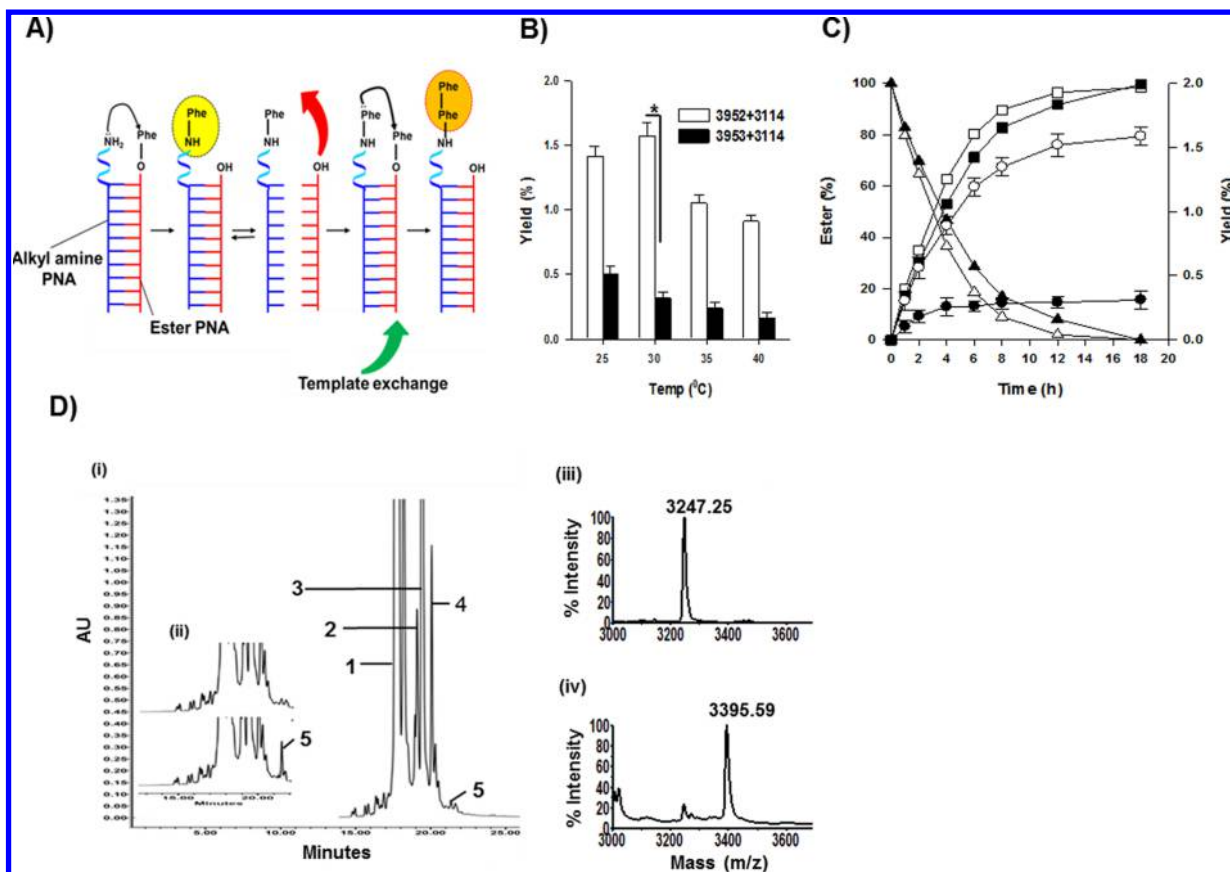


Figure 4. (A) Scheme of the intermolecular phenylalanine transfer in the binary PNA duplex system. The peptide product can be further extended through template exchange as exemplified by dipeptide formation (orange circle). (B) Effect of temperature on the intermolecular phenylalanine transfer. Reactions were carried out with an equimolar ratio between the ester PNA 3114 and a fully match (PNA 3952) or mis-match (PNA 3953) duplex under the above experimental conditions (* $p < 0.05$). Experiments were performed in triplicate, and yields were expressed as percent (%) yield (bar represent standard error of the mean). (C) Time kinetics of ester hydrolysis and phenylalanine transfer. The left axis shows the fraction of intact PNA 3952 (open triangle) and PNA 3953 (filled triangle) ester and that of hydrolyzed PNA 3952 (open square) and PNA 3953 (filled square), while the right axis shows the product yield of PNA 3952 (open circle) and PNA 3953 (filled circle) obtained by HPLC analysis. (D) Peptide and dipeptide formation. Reactions were carried out with an equimolar ratio between the ester PNA 3114 and fully match (PNA 3952) or mis-match (PNA 3953) in a mixture of 50% NMAA in 50 mM carbonate buffer, pH 10 at 30 °C. A fresh supply of 1 mM ester PNA 3114 was supplemented every 2 days and the reaction was allowed to run for 7 days. (i) HPLC analysis of the reaction mixture of PNA 3952 and PNA 3114. (ii) As part i, with coinjected chemically synthesized authentic product (synthesized by the PNA 3954 and PNA 3114). HPLC analysis of the reaction mixture allowed the detection of one extra well separated peak (peak 5), and MALDI-TOF analysis of this peak showed a mass of $m/e = 3395$, corresponding to that expected for the dipeptide product. Peak 1 corresponds to amino alkylamide PNA 3952 (with eg_1 linker), peak 2 corresponds to intact ester PNA 3114, peak 3 corresponds to hydrolyzed ester PNA (–Phe), peak 4 corresponds to transfer product (Phe-transfer) as established by MALDI-TOF mass spectrometric analysis of purified product. (iii) Transfer product (Phe-transfer). (iv) Dipeptide product (–Phe–Phe–).

these conditions the Phe-transfer is predominantly occurring as an intramolecular, interstrand reaction at the end of the PNA duplex and that it is therefore directed by the PNA–PNA sequence recognition. Accordingly, a kinetic time course experiment (Figure 3D, Supporting Information Table S7) likewise showed that the estimated initial reaction rate of Phe-transfer for the matched PNA 3751 was higher than that of the mismatch PNA 3871.

It has been shown that metal cations³¹ and also the dipeptide seryl-histidine (Ser–His)³² may catalyze peptide bond formation, and we therefore investigated the effect of different metal cations (Mg^{2+} , Mn^{2+} , Cu^{2+} , and Zn^{2+}) and the dipeptide (Ser–His) on the yield of the Phe-transfer for fully match (PNA 3751) and mismatch (PNA 3871) hairpins (Figure 3C). Whereas the presence of some metal cations particularly Mg^{2+} , Mn^{2+} , and Cu^{2+} significantly increased the yield, no increase was observed with Zn^{2+} ions or the dipeptide seryl-histidine

(Ser–His). Inclusion of Mn^{2+} at 0.1 mM yielded 3-fold increase and Mg^{2+} and Cu^{2+} at 0.1 mM and 0.01 mM, respectively, resulted in a 2-fold increase. No enhancement was seen at higher concentrations, and for Cu^{2+} and Zn^{2+} , the yield actually decreased most likely due to preferential catalysis of the ester hydrolysis at these concentrations (Supporting Information Table S3).

Intermolecular Phenylalanine Transfer Using a Binary PNA Duplex System. A series of PNA constructs (PNAs 3382–3387, 3952; Table 1) were synthesized for an analogous binary PNA duplex system (Figure 4A). Using identical experimental conditions as for the hairpin stem loop system, a *bona fide* phenylalanine transfer reaction product could be identified by HPLC and MALDI-TOF analysis (Supporting Information Figure S10). In order to establish whether product formation (Phe-transfer) was indeed dependent on duplex formation, we performed a temperature study using a mismatch

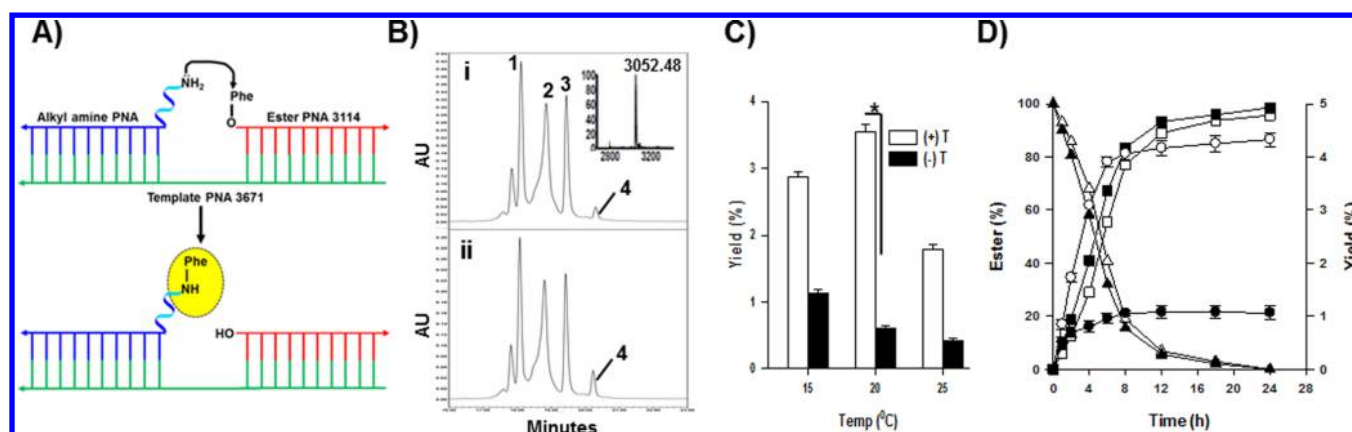


Figure 5. (A) Scheme of intermolecular phenylalanine transfer in the ter-molecular PNA template assisted system. (B) HPLC and mass spectrometry analysis. Using an equimolar ratio between all three PNA components (amino alkyl amide PNA 3858 (with C8 linker), ester PNA 3114 and template PNA 3671) in a mixture of NMAA (20%) in 50 mM carbonate buffer, pH 10 at 20 °C for 24 h. (i) HPLC analysis of the reaction mixture of PNA 3952 and PNA 3114. (ii) As in part i, with coinjected chemically synthesized authentic product: Peak 1 corresponds to amino alkyl amide PNA 3858, peak 2 corresponds to template PNA 3671, peak 3 corresponds to hydrolyzed ester PNA (–Phe) and peak 4 corresponds to transfer product (Phe-transfer) as supported by MALDI mass spectrometric analysis of purified product (Inset picture). (C) Effect of temperature on the intermolecular phenylalanine transfer. Reactions were carried out with template PNA 3671 (+T) and an equimolar ratio between the amino alkyl amide PNA 3858 (with C8 linker), or without template (–T) under the above experimental conditions at different temperature (* $p < 0.05$). Experiments were performed in triplicate and yields were expressed as percent (%) yield (bar represent standard error of the mean). (D) Time kinetics of ester hydrolysis and phenylalanine transfer. The left axis shows the fraction of hydrolyzed (+T, open square; –T, filled square) and intact (+T, open triangle; –T, filled triangle) ester PNA 3114 (open triangle); while the right axis shows Phe-transfer product yields (+T, open circle; –T, filled circle) obtained by HPLC analysis.

PNA (3953) as control (Figure 4B). These results show that the fully matched binary duplex gives significantly higher yield up to 5-fold compared to the mis-match one at all tested temperatures and that the highest yield was obtained at 30 °C. A more than 5-fold higher yield, and similarly faster initial reaction rate (Supporting Information Table S7) than those of the mis-match system (Figure 4B and C), was seen, thereby very strongly supporting an interstrand Phe-transfer in the PNA duplex.

The “hairpin PNA” data, showed that metal cations particularly Mg^{2+} , Mn^{2+} , and Cu^{2+} significantly increased the product yield (Phe-transfer), and similar experiments were performed with the binary duplex system (Supporting Information Figure S11). We observed that 0.1 mM of Mn^{2+} or dipeptide (Ser–His) gave very limited (up to 30%) increase in the transfer yield of fully match binary duplex, while the other metal cations showed no effect. For the mis-match binary duplexes, no effect of the ions at 0.1 mM was observed on the transfer yields, but a further increase in the concentration of metal cations reduced the transfer yields. This decrease can be partially explained by enhanced rate of ester hydrolysis as exemplified in Supporting Information Table S3. The effect on Phe-transfer of metal cations and the dipeptide Ser–His was not as pronounced as seen in the hairpin system.

Dipeptide Formation. Duplex exchange could in principle lead to PNA coupled Phe–Phe dipeptide formation. However, under the above used reaction conditions, no such product was detected. Therefore, we attempted to prolong the half-life of the ester as well as increase duplex strand exchange kinetics (by lowering the stability of the duplex) by increasing the NMAA content. T_m studies of binary PNA duplexes showed that 50% NMAA gives approximately 70% duplex for the fully match system at 30 °C. Thus, 50% NMAA was used, and additional ester was supplemented every second day (Figure 4D). The reaction mixture was analyzed after 7 days and a small amount of dipeptide product could be detected and identified by HPLC

and MALDI mass spectrometry analyses (Figure 4D: i, iii, and iv). The identity of the dipeptide product was further verified by coinjection HPLC analysis using independently synthesized product (Figure 4D,ii). As anticipated, the dipeptide product was formed in much lower yield (<1%) than that of the primary Phe transfer product (~10%), and dipeptide formation was not detected using the mis-match duplex.

Phenylalanine Transfer Using a Ternary PNA Template Guided Systems. We finally constructed and characterized a three component system where two PNA oligomers were assembled on a PNA template (Figure 5A), thereby primitively mimicking the features of tRNA/mRNA/ribosomal translation. In order to maximize the possibility of Phe-transfer, the distance between the primary amine of an amino alkyl amide PNA (acceptor) and the thymine PNA ester (donor) was varied by using different linker length moieties (PNA 3852–3858; Table 1). The formation of the Phe-transfer product was confirmed by HPLC coinjection and MALDI-TOF analysis (Figure 5B). Furthermore, the effect of temperature was studied (Figure 5C). The reaction was clearly enhanced by the presence of the template PNA strand at all tested temperatures, and the highest yield (3.5%) and most significantly the highest template dependency (7-fold increase) and reaction rate (Figure 5D, Supporting Information Table S7) was observed at 30 °C. Somewhat surprisingly, in view of the close proximity of the two ends in the ternary complex, the longer C8 linker length (PNA 3858) gave the highest yields (~4%) (Supporting Information Figure S12), and addition of metal cations (Mg^{2+} , Mn^{2+} , Cu^{2+} , or Zn^{2+}) or the dipeptide Ser–His had modest effect on the Phe-transfer reaction (Supporting Information Figure S13). Analogously to the binary duplex system, the presence of Mn^{2+} or dipeptide (Ser–His) at 0.1 mM gave very limited (up to 30%) increase in the transfer yield, while the other metal cations showed no effect. A further increase in the concentration of metal cations reduced the transfer yields.

Discussion. The present results clearly demonstrate that sequence instructed phenylalanine transfer from a simple aminoacyl ester donor on one PNA strand to an amino acceptor on a hybridized PNA strand can take place in aqueous solution at slightly alkaline pH in a process that primitively mimics biological peptide bond formation in biological peptide synthesis. It is noteworthy that in the PNA system no anchimeric assistance via a vicinal hydroxyl group is possible, as is the case in 3'-charged tRNA in biological translation, which was found to be a prerequisite in the mini-helix RNA translation mimics studied by Schimmel and co-workers.^{5,6} This finding has implications for possible prebiotic evolution of nucleic acid instructed peptide synthesis (translation) as it shows that this process may not have required RNA, and thus from a chemical point of view may have occurred in a pre-RNA world, based on another genetic material such as PNA, TNA,^{33,34} or GNA.³⁵

Furthermore, we note that the efficiency of the PNA directed acyl transfer in terms of yield is quite low and that this to a large extent is limited by the high rate of hydrolysis (low stability) of the ester relative to amide formation. Therefore, shielding of the ester from water would increase ester half-life and consequently "translation" efficiency (yield) as observed by including organic solvent (DMSO, NMAA) in the medium. Indeed, a parallel may be drawn to contemporary ribosomes in which a pocket of reduced water activity at the peptidyl transferase center is formed.³⁶ Also, it has been shown that the catalytic effect of the ribosome is overwhelmingly entropic in origin, suggesting that the ribosome enhances the rate of peptide bond formation mainly by positioning the substrates and/or by desolvation within the active site, rather than by direct chemical catalysis.³⁷

Somewhat unexpectedly we observed only limited enhancement by (some) divalent cations (Mn^{2+} in particular). This is ascribed to their competing catalysis of ester hydrolysis. Therefore, by proper coordination of metal ions at the "reaction center" in combination with shielding from water, preferential catalysis of acyl transfer should be enhanced. Indeed, further development of the PNA hairpin system may be exploited to study both the effect of water shielding as well as of specific metal ion catalysis, by building metal coordinating ligands onto the PNA. Thus, although the ribosomal structure is known in great detail, the mechanism of peptidyl transferase catalysis is still not fully understood.³⁸ However, it is clear that ribosomal proteins are not directly involved and that proper structural positioning of the reactants by nucleobase residues of the rRNA forming an intricate hydrogen bonding network, also including water molecules, in a cavity shielded from bulk water is critical.³⁸ In principle, it should be possible to expand the simple PNA systems presented here to include PNA "aptamer" (or peptide) loops, which analogously could mimic (some of) these functions of the ribosome.

In conclusion, we have identified conditions which allow PNA hybridization (template) assisted intermolecular aminoacyl transfer from one PNA strand to another PNA strand using simple aminoacyl ester chemistry analogous to biological peptidyl transferase reactions but without the anchimeric assistance from the ribose and without ribosome catalysis. Although yields were low (<10%), these results help define the minimum chemical boundary conditions for the translation process, which Nature has optimized through biological evolution, and also give insight into the possibilities for the prebiotic emergence of translation as a target for evolution. If it

is eventually possible to make a case for preRNA translation evolution, one may argue and speculate that the significance of an RNA world would be less prominent as it would then comprise an evolutionary step from a "life form" already capable of "nucleic acid" replication as well as of peptide formation by translation.

In relation to the prebiotic origin of life, the present results clearly show that nucleobase sequence directed peptide synthesis based on simple aminoacyl esters (as present in contemporary life) requires protection of the ester from hydrolysis and/or significant enhancement (catalysis) of amide bond formation in order to obtain sufficient efficiency for evolution. It may be speculated that mineral surfaces of which many have been proposed as possible catalysts for prebiotic chemical reactions³⁹ could also serve as catalysts for the ester based peptide bond formation as well as shield against water upon adsorption of the (PNA) oligomers, and likewise, tidal ponds drying out will reduce water activity. Future studies will reveal whether such systems and/or conditions can be discovered.

METHODS

PNA monomers⁴⁰ and thymine L-phenylalanine ester PNA monomer synthesis are reported in Supporting Information. PNA concentrations were determined using molar extinction coefficients: ϵ_{260} of adenine = $15400 \text{ M}^{-1} \text{ cm}^{-1}$, ϵ_{260} of guanine = $11700 \text{ M}^{-1} \text{ cm}^{-1}$, ϵ_{260} of thymine = $8800 \text{ M}^{-1} \text{ cm}^{-1}$, and ϵ_{260} of cytosine = $7400 \text{ M}^{-1} \text{ cm}^{-1}$ at 65°C . The Phe transfer reaction product was detected and identified by HPLC and MALDI-TOF analysis and product yield (%) were calculated.

HPLC analysis was performed on a Waters Symmetry 300 C18, $3.9 \times 150 \text{ mm}$ ($5 \mu\text{m}$ particles, 100 \AA pore size) analytical column equipped with a Zorbax Eclipse XDBC18 ($5 \mu\text{m}$ particles, 80 \AA pore size) guard column (Agilent) at $\lambda = 260 \text{ nm}$. Solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile) were used in a linear gradient of 0–50% (0–35 min) at 50°C with a flow rate of 1 mL/min .

MALDI-TOF/MS analysis was performed on a Voyager-DE Pro bio spectrometry workstation (PerSeptive Biosystems) equipped with a 337 nm nitrogen laser and operating in reflector mode (200 laser shots; 20 kV).

T_m measurements were done at 260 nm using a Cary 300 Bio UV–visible spectrophotometer (Varian, Cary, NC, U.S.A.) connected to a temperature controller. Equimolar mixtures of complementary PNA strands were dissolved in 50 mM buffer (phosphate or carbonate buffer, pH 7–9) with desired amount of organic cosolvent. Thermal melting (T_m) profiles were obtained using heating–cooling cycles in the range from $+5$ to 95°C with a rate of 0.5°C/min . T_m was determined from the peak of the first derivative of the heating curve (1.0 cm path length, 1.0 mL). T_m values at $>60\%$ DMSO and NMAA could not be obtained because loss of the upper baseline.

Ester Hydrolysis Kinetics. The stability of Phe–ester PNA 3114 ($100 \mu\text{M}$) was measured in 50 mM CHES or carbonate buffer at different pH (7.5–9) and temperatures (25 – 45°C) with inclusion of organic cosolvent (NMAA and DMSO). PNAs were analyzed and quantified by HPLC analysis. First-order rate constants (k) were obtained by plotting the natural logarithm of the inverse of the remaining fraction of ester PNA against time and the half-life ($t_{1/2}$) was calculated ($t_{1/2} = 0.693/k$) from these.

Ethyl Acetate and Phenylalanine Methyl Ester Acyl Transfer Reaction. Ethyl acetate acyl transfer were carried out with 1 mM glycine alkyl amide PNA 3382 and ethyl acetate ester (30%) in a mixture of DMSO (60%) with a variety of buffers (50 mM CHES, carbonate, or imidazole) at pH 9.0, 60°C for 12 h . For phenylalanine methyl ester, acyl transfer were carried out with a hundred fold excess of the hydrochloric salt of phenylalanine methyl ester in a mixture of DMSO (90%) in carbonate buffer, pH 9.0 at 60°C for 24 h .

Phenylalanine Interstrand Transfer With in a Hairpin PNA Duplex System. Phenylalanine (Phe) intramolecular transfer was

carried out with hairpin PNA constructs at 1 mM in a mixture of organic solvent (DMSO or NMAA) in carbonate buffer at desired pH and temperature for 24 h. The effect of different metal cations and the dipeptide (Ser–His) at various concentrations (0.01 to 1 mM) was examined at pH 10, 30 °C.

Intermolecular Phenylalanine Transfer Using Binary PNA Duplex System. Using an equimolar ratio (1 mM each) between binary PNA duplexes (alkyl amide PNA with varying linker moieties, (PNA 3382–3387, PNA 3952–3953) and ester PNA 3114) a mixture of NMAA (20%) in 50 mM carbonate buffer, pH 10 at different temperatures for 24 h. The effect of different metal cations and the dipeptide (Ser–His) at various concentrations (0.01 to 1 mM) was examined at pH 10, 30 °C.

Dipeptide Formation Using Binary PNA Duplex System. An equimolar ratio (1 mM each) between ester PNA 3114 and fully match (PNA 3952) or mis-match (PNA 3953) in a mixture of 50% NMAA in 50 mM carbonate buffer, pH 10 at 30 °C was used. A fresh supply of 1 mM ester PNA 3114 was added every 2 days (three additions in total) and the reaction was run for 7 days.

Phenylalanine Transfer Using a Ternary PNA Template Guided Systems. An equimolar ratio between all the ternary complex components (alkyl amide PNA with varying linker moieties, ester PNA 3114 and template PNA 3671) in a mixture of NMAA (20%) in 50 mM carbonate buffer, pH 10 at 20 °C for 24 h. The effect of different metal cations and the dipeptide (Ser–His) at various concentrations (0.01 to 1 mM) was examined as above at 20 °C.

■ ASSOCIATED CONTENT

■ Supporting Information

Supplementary data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +45 35327762. Fax: +45 35396042. Email: ptrn@sund.ku.dk.

Present Addresses

^{||}Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan 48109-5622, U.S.A.

[⊥]Chiesi Farmaceutici Largo Francesco Bellolli 11/A, 43122 Parma, Italy

Notes

The authors declare no competing financial interest.

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